Original Research Article

Fertilization With Silicon in Sweet Pepper improved Plants Grown Under Salt Stress

ABSTRACT

Aims: The objective of this research was to investigate the effect of calcium silicate on gaseous exchanges and production factors in the sweet pepper, cultivated under conditions of soil salinity induced by potassium fertilization, in protected cultivation.

Study design: The experiment was arranged in a randomized complete block design in a 2 × 5 factorial scheme with five replications.

~~Place~~ **Place and Duration of Study:** The experiment was conducted in the sector of Olericultura and Experimentation of the course of Agronomy from October 12, 2018 to February 2019.

Methodology: The experiment was arranged in a randomized complete block design in a 2 × 5 factorial scheme (two sources of correction: limestone and silicon and five increasing doses of KCl equivalent to 150, 300, 450, 600 and 700 kg ha^{-1} of K_2O). T~~t~~en treatments with five replicates where each experimental unit consisted of a polyethylene pot, with a volume of 19 dm^3. The electrical conductivity, the determination and quantification of silicon in soil and plant, liquid photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpiration, water use efficiency and instantaneous carboxylation efficiency were analyzed.

Results: With the increase of K_2O in the soil there was a tendency of reduction in liquid photosynthesis, transpiration, stomatal conductance, intercellular CO_2 concentration, water use efficiency and instantaneous carboxylation efficiency in the presence and absence of calcium silicate. Higher doses of K_2O (300, 450 and 600 kg ha^{-1}) reduced the length and yield of sweet pepper fruits in the presence and absence of calcium silicate. The dose of 150 Kg K_2O favored the growth of sweet pepper plants in the presence of calcium silicate.

Conclusion: In the culture of sweet pepper in protected cultivation, the increase in the rates of potassium fertilization increases the electrical conductivity of the soil and the silicon content in the leaves. Doses up to 300 kg K_2O ha^{-1} increase the biometric factors of production. High doses of K_2O reduce gas exchange and water use efficiency, and the application of calcium silicate in the soil attenuates these effects.

***Keywords**: Abiotic stress; photosynthesis; Capsicum annuum; salinization; calcium silicate.*

1. INTRODUCTION

Brazil is among the main sweet pepper producing countries [1]. The main sweet pepper producing states in Brazil are Minas Gerais, São Paulo, Ceará, Rio de Janeiro, Espírito Santo and Pernambuco (87% of the total) [2]. It is possible to produce sweet peppers all year round, but it develops better in the summer [3]. Currently, sweet pepper producers have preferred to cultivate this crop in a protected environment, which allows a continuous supply and harvesting in periods of low supply of the product in the market, thus achieving more competitive prices [4].

In the State of São Paulo, in 2018, about 65.800 tons of sweet pepper were produced in 2.560 ha~~$^{-1}$~~ [5]. In the production of vegetables in protected cultivation, it was verified that, after three years of cultivation, many producers do not obtain adequate productivities and quality of the fruits, because there are many problems related to excessive fertilization, leading the soil to an accumulation of salts. The losses suffered by the producers are generated by improper practices of the incorrect management of the fertilization in greenhouse [6]. Therefore, the symptoms of these anomalies in plants under conditions of nutritional imbalance are common, due to the saline stress of the soil solution [3]. Although irrigation water in protected crops is of good quality, the addition of fertilizers, when using the fertigation technique, makes it saline, increasing the risk of soil salinization [7].

Potassium (K) is a nutrient demanded in great quantity by the culture of the sweet pepper, being the main source used by the producers is potassium ~~chloride, that~~chloride that has high saline index, being one of the main sources of salinization of the soil in cultivation. Potassium sulfate has a salt content equivalent to half of the salt content of potassium chloride, which makes it more suitable for soils with tendency to salinization [8].

The exogenous application of silicon (Si) significantly improves the development of plants under conditions of salt stress [9]. Calcium silicate can be used as a corrective of soil acidity, neutralizing exchangeable aluminum, providing nutrients to the plant and increasing soil base saturation [10]. When saline stress occurs, there is a decrease in the relative water content in the leaf, indicating that the plants are exposed to osmotic stress [11]. Studies have shown that Si increases the relative water content in plants under conditions of salt stress [12], decreasing the toxicity of the salts to the plant and improving its growth [13], increasing the thickness of the leaves, due to deposition of Si, which reduces transpiration and decreases water loss [14].

Due to the condition of soil salinization, nutritional disorders may occur, inducing antagonistic relationships between nutrients in the plant, which significantly reduces crop yields [15]. Elevation of K content in soil can induce nutritional imbalance for plants [16]. However, it is necessary to know the effects of the interactions between saline stress and the use of silicon in the culture of sweet pepper that has been cultivated in protected culture.

The objective of this work was to investigate the effect of calcium silicate on gaseous exchanges and production factors in the sweet pepper, cultivated under conditions of soil salinity induced by potassium fertilization, in protected cultivation.

2. MATERIAL AND METHODS

The experiment was conducted in the sector of Olericultura and Experimentation of the course of Agronomy from October 12, 2018 to February 2019 in greenhouse. A protective structure model was used, with 225 meters each (9 meters wide by 25 meters long) and right foot of 4 meters. The structure was covered with agrofilm, of blue color. The sweet pepper cultivar Magali R. was used. The seedlings were produced in trays with 128 cells, 6.0 to 6.2 cm high, with substrate composed of inert material and free of pathogens. Transplanting was carried out on November 20, 2018 using a seedling per pot, when they had three to four definitive leaves, which occurred around 35 days after sowing.

The experiment was arranged in a randomized complete block design in a 2 × 5 factorial scheme (two sources of correction: dolomitic limestone and silicon and five increasing doses of KCl equivalent to 150, 300, 450, 600 and 700 kg ha^{-1} of K_2O. It was applied 1.62 ha^{-1} Mg of dolimite limestone with 80 % total neutralizing power (45 % CaO and 10% MgO) corresponding to 15.39 g pot and 1.87 Mg ha^{-1} of calcium silicate with total neutralizing power 86% (40.7% SiO_2 and 10% CaO) corresponding to 17.85 g by pot, the source $CaSiO_3$ used was reagent pure for analysis. Whose treatments and potency equivalence are described in Table 1~~.~~ Each experimental unit consisted of a 19 dm^{-3} polyethylene pot filled with Oxisol [17], after incubation of limestone and calcium silicate, fertilization was per-formed for the macro and micronutrients following the recommendation of [18] and [19] adapted for experiments conducted in pots and for the corn crop.

The soil was classified as Oxisol [20] and samples were collected at a depth of 0-20 cm. The samples were placed to dry, crushed through a 5-mm sieve and mixed to describe the chemical and physical compositions. Chemical and physical compositions of the soil used in this study, according to [21], were: pH in water (1:2.5)= 5.2; level of organic matter (OM)= 1.42 (dag kg^{-1}); P and K by Mehlich I extraction = 3.69 and 30.41 (mg dm^{-3}); Mg, Ca and Al extractable by 1 M KCl solution= 7.59, 1.12 and 0.20 (cmol dm^{-3}); Si= 3.29 (mg dm^{-3}); Zn= 1.05 (mg dm^{-3}); Cu= 1.38 (mg dm^{-3}); S= 13.24 (mg dm^{-3}); B= 0.07 (mg dm^{-3}); Fe= 53.62 (mg dm^{-3}); T = cation exchange capacity at pH 7.0 (3.62 %); t= cation exchange capacity effective (5.02 %); m = aluminum saturation index (12.50 %); V = Base saturation index (27.85 %). Soil granulometry was the soil physical composition used in this study, determined by the pipette method (sand, silt and Clay = 60 %, 11 % and 29 %). After incubation of limestone and calcium silicate, fertilization was performed for macro and micro-nutrients following the recommendation of [18] and [19] adapted for experiments conducted in pots for sweet pepper crops. The soil chemical analysis was done at the soil science laboratory of the Federal University of Lavras, Brazil. The pots had holes in the bottom where a layer of 0.30 m of folded sombrite was placed to avoid soil loss and to allow drainage of excess water, if it occurred.

Before the transplanting of the crop, 300 mg dm^{-3} of urea (45 % N), 300 mg dm $^{-3}$ of simple superphosphate (18 % P_2O_5) was applied and incorporated into the soil, pure reagent was used for analysis for both fertilizers. The calculations for soil correction were based on recommendations [18]. For N, the equivalent of 12.22 g of urea per pot was divided into three applications and, for P_2O_5, 72.52 g of simple superphosphate per pot applied at planting was used. Coating fertilizations started at 15 days after transplant (DAT) and were performed biweekly. The basic fertilization for K_2O was made with KCl using pure reagent source for analysis (60 % K_2O), as described in Table 1. After the application of the fertilizer, the soil was moistened for 35 days to favor the chemical reaction of the corrective and fertilizer. The pots were distributed at spacing of 0.63 m between plants and 1.0 m between rows.

Table 1. Treatments and equivalence in pots based on the two correctives (calcium silicate and dolomitic limestone) and doses of K_2O.

Treatments	Corrective		K_2O doses kg ha^{-1} of K_2O	
T1	Calcium silicate	-	150	-
T2	Calcium silicate	-	300	-
T3	Calcium silicate	-	450	-
T4	Calcium silicate	-	600	-
T5	Calcium silicate	-	700	-
T6	-	Calcário	-	150
T7	-	Calcário	-	300
T8	-	Calcário	-	450
T9	-	Calcário	-	600
T10	-	Calcário	-	700

The water characterization of the soil was determined by its water retention characteristic curve (Fig.1). The parameters of the soil water retention curve used in irrigation ~~irrigation~~ and irrigation management were obtained based on the model proposed by [22], with the aid of the Solver application of Microsoft Office Excel® software ($\theta = 0.4215 \times [1 + (0.2040 \times |\Psi m|)^{1.8757}]^{-0.4669} + 0.2670$), where: θ = current moisture $cm^3.cm^{-3}$ and Ψm = stress, kPa. The field capacity was estimated to be equivalent to the voltage and humidity at the inflection point of the retention curve, as proposed by [23]: $\Psi m = 1 / \alpha \, [1 / m]^{1/n}$, where: Ψm = tension at the inflection point of the curve, kPa; α, m and n = adjustment parameters of the model equation proposed by [22]. The moisture value in the field capacity found was 0.3458 $cm^3.cm^{-3}$ for a voltage of 4.25. Soil moisture was determined through tensiometers, using the water potential of -35 kPa, considered as adequate for the development of the crop [24].

The irrigation was done by drip irrigation; the self-compensating emitters being manually inserted in polyethylene hoses. The calculation of the operating time of the irrigation system was made based on the humidity sensors (tensiometers) installed in the depth of 0.15 m. With the observed stresses, the corresponding moisture values were estimated from the water retention curve in the soil.

With these moistures and the one corresponding to -30 kPa [19] and, considering the effective depth of the root system (0.15 m), the net and gross replacement slides were calculated for the treatments. Aiming at the replacement of soil water, two readings were performed daily in the tensiometers, one in the morning (8:00 am) and one in the afternoon (14:00 pm).

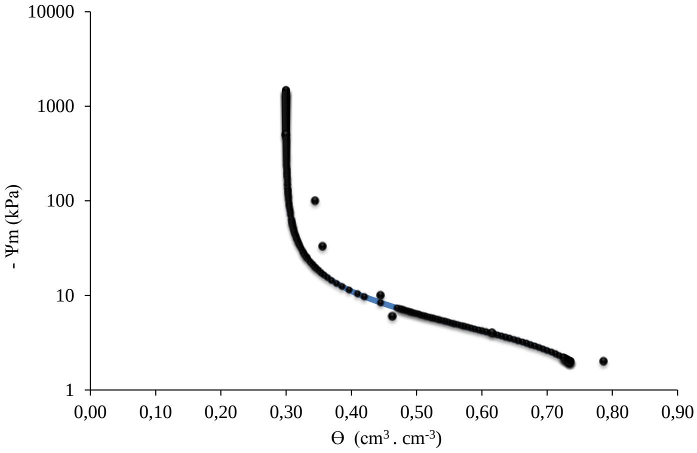

Fig. 1. Water retention characteristic curve of the Oxisol used in the experiment.

At the end of the experiment, the electrical conductivity (EC) was determined in the saturated paste extract [25], which is the method used as reference for EC determination and adopted in various regions of the world. To do so, the soil passed through the 2 mm sieve and allowed to stand for 24 h to air dry. Afterwards, 800 g of soil were added in plastic containers, with capacity for 1200 mL, with 500 mL of distilled water added. After the mixture turned into a paste, the container was covered with foil remaining for 24 h. After this time, the slurry was again stirred, standing for 1 h. By means of the vacuum filtration of the saturation paste, the solution of the soil was extracted, after which the EC reading was measured. The electrical conductivity of the saturated pulp was corrected considering the soil water retention characteristic using a digital conductivity meter (Lutron, model CD-4303).

For the quantification of the silicon in the soil, soil samples were taken from the pots grown with sweet pepper and prepared for analysis. The samples were dried at room temperature (TFSA) and subsequently sieved (<2,0 mm). The extraction procedure was performed to maintain the same soil: solution ratio, that is, for each 10 g of soil, 100 mL of extractor was added. The extractors used were: Acetic acid 0,5 mol L^{-1} [26]: 100 mL of 0.5 mol L^{-1} acetic acid was added to a 150 mL plastic flask containing 10 g de soil. The plastic bottle was capped and shaken horizontally for one hour. After 30 minutes, the extract was filtered (plastic funnel), using filter paper number 42; Buffer pH 4.0: 100 mL of a buffered solution at pH 4.0 acetic acid plus sodium acetate (49.2 mL of concentrated acetic acid and 14.800 g of anhydrous sodium acetate were dissolved in 1,0 liter of distilled water, and the pH adjusted to 4.0 with the addition of acetic acid) were added in a 150 mL plastic flask with 10 g soil and shaken horizontally for one hour. The vials were then held for 30 minutes and then the plastic funnel extract and filter paper number 42 filtered; Calcium chloride 0.0025 mol L^{-1} [27]: 100 mL of a 0.0025 mol L^{-1} calcium chloride solution was added in a plastic flask containing 10 g of soil. Thereafter, it was shaken horizontally for 15 minutes and then decanted from overnight. The following day, the extracts were filtered (plastic funnel and filter paper number 42); Water: 100 mL of distilled and demineralized water were added in 150 mL plastic bottles with 10 g of soil. Henceforth, the procedure was the same as for acetic acid.

The determination of Si in the extract was made by mixing 10 mL of the extract (filtrate / decanting) in 1 mL of sulfo-molybdenum 7.5% solution (7.5 g ammonium molybdate in 10 mL + ac. sulfuric 9 mol L^{-1} in 100 mL). After 10 minutes 2 mL of the 20% tartaric acid solution was added and after 5 minutes 10 mL of the 0.3% ascorbic acid solution was added. After one hour, the Si was read in a spectrophotometer and at the wavelength of 660hm. The quantification of silicon in the leaves was performed by the colorimetric method of molybdenum blue in the laboratory of mineral nutrition of plants in the Laboratory of Mineral Nutrition of Plants of the Federal University of Uberlandia, Brazil [28].

The shoot dry matter (leaves + stem) was collected at 120 days after transplanting (DAT), to determine dry shoot mass (MMSPA). To dry the material an oven was used at 70 °C with forced ventilation until constant mass was reached. The shoot + stem was processed together. The heights of the plants (m) were evaluated with the help of a scale, measuring the distance between the base of the plant collar to the end of the main stem, the production, which was determined throughout the reproductive stage of the plants, and also the diameter, length, weight and diameter of commercial fruits.

For the analysis of liquid photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpiration, water use efficiency and instantaneous carboxylation efficiency, the IRGA model LI-6400XT, (Li-Cor, Lincoln, Nebrasca, USA) was used. Two plants of each cultivar were chosen randomly, being defined as the sample unit the sixth leaf from top to bottom, fully expanded and mature. Because it is a species with a composite leaf, the first three leaflets of each leaf were used to measure, totaling six measurements. The value of 850 µmol m^{-2} s^{-1} of saturation irradiance, defined by the realization of a light curve, was set using the value of radiation that induced the maximum photosynthesis. Sweet pepper is a C3 plant, where a cyclic mechanism of enzymatic reactions converts CO_2 into carbohydrates through the reductive photosynthetic cycle (C3), generating the 3 phosphoglycerate. Therefore, IRGA camera temperature was controlled at 28 °C, since in C3 plants the maximum rate of photosynthesis is reached at relatively low radiation intensity, causing no destruction or damage to the photosynthetic apparatus. Measurements were performed on a 6 cm^2 sheet area.

The results found in the different evaluations were submitted to analysis of variance. For the evaluation of the means, the Scott-Knott or t-test were applied, according to the theories recommended by [29]. The standard deviations were calculated and the correlation estimators (Pearson or Spearman) were used, using SISVAR software [30].

3. RESULTS AND DISCUSSION

The electrical conductivity (EC) of the soil (Fig. 2) increased with increasing doses of K_2O in both correctives (calcium silicate and dolomitic limestone). The EC of 2.76 and 2.16 dS m^{-1} were the ones that provided the greatest vegetative development and production, these results agree with those found by [31], who studied the influence of EC on eggplant concluded that the EC of 2.36 dS m^{-1} provided the greatest development and fruiting. The higher dry matter yield of roots, stems, leaves and fruits in eggplant plants was obtained with EC of nutrient solution of 2.10 dS m^{-1} [32]. The use of a dose greater than 60 kg $ha^{-1}$of K_2O may cause some damage to the legumes due to its saline effect, which may have occurred in this experiment with doses greater than 100 kg $ha^{-1}$of K_2O [33]. The electrical conductivity increased linearly with the increase of the KCl dose applied in two sources of potassium fertilization, due to the increase of the electrolytic concentration of the soil solution, which is proportional to the increase in the concentration of ions in the solution [34].

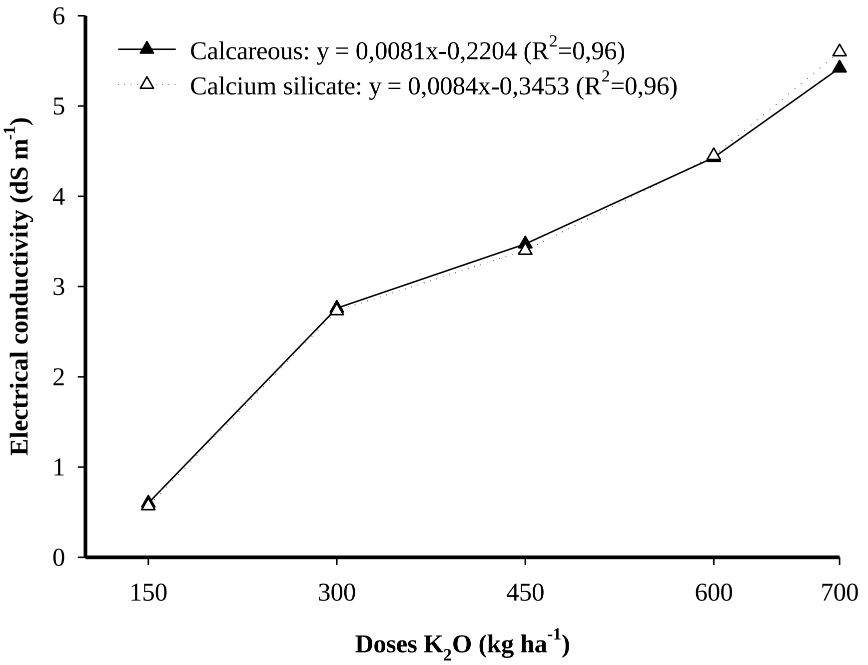

Fig. 2. Electrical conductivity of the soil as a function of the K_2O doses and sources of correctives (calcium silicate and dolomitic limestone).

The concentration of Si in the soil did not vary in the different doses of K_2O studied when calcium or calcium silicate was applied (Table 2). However, in the interaction between the doses of K_2O x sources of correctives it was observed that the silicon concentration was higher for the treatment using calcium silicate, due to the fact that it is a soluble source of Si.

Table 2. Soil silicon content in $CaCl_2$ 0.01 mol L^{-1} as a function of K_2O doses and corrective sources (dolomitic limestone and calcium silicate).

K_2O doses	**Calcium silicate**	**Dolomitic limestone**
(kg ha^{-1})	**Content tho Si (mg kg^{-1})**	
150	6.00 Aa	5.00 Ab
300	5.70 Aa	5.00 Ab
450	5.80 Aa	4.80 Ab
600	5.75 Aa	5.00 Ab
700	6.00 Aa	5.20 Ab

Capital letters equal in the column, do not differ at the level of significance of 5%; Minor letter in the same line, do not differ at the level of significance of 5%.

For the silicon content in the sweet pepper leaf (Table 3) differences were observed between the doses of K_2O. When the calcium silicate was applied, the highest levels were found with 600 and 700 kg ha^{-1} K_2O. As for the interaction between the correctives (calcium silicate x dolomitic limestone), independent of the K_2O dose, the higher silicon contents were found when calcium silicate was applied.

Table 3. Silicon content in the leaf (%) as a function of K_2O doses and corrective sources (dolomitic limestone and calcium silicate).

K_2O doses (kg ha^{-1})	Calcium silicate	Dolomitic limestone
	Content tho Si (%)	
150	13 Ca	12 Ab
300	14 Ca	12 Ab
450	18 Ba	13 Ab
600	20 Aa	13 Ab
700	20 Aa	14 Ab

Capital letters equal in the column, do not differ at the level of significance of 5%; Minor letter in the same line, do not differ at the level of significance of 5%.

With increasing doses of K_2O in the soil there was a tendency of reduction in the liquid photosynthesis (Fig. 3A), transpiration (Fig. 3B), stomatal conductance (Fig. 3C), intercellular CO_2 concentration (Fig. 3D), water use efficiency (Fig. 3E) and instantaneous carboxylation efficiency (Fig. 3F), in the presence and absence of calcium silicate. However, it was observed that with the application of calcium silicate all these variables presented higher values. The deposition of silicon in plant tissues improves the interception of light and decreases transpiration [35]. Increased availability of Si favors increased productivity, since Si can act indirectly in photosynthetic and biochemical processes, especially when the plant is subjected to some type of stress [36]. The translocation of silicon from the roots to the aerial part of plants may be related to the increase in photosynthetic capacity, greater resistance to possible damage and reduction in the evapotranspiration process, which, consequently, improves the use of available water in the soil [37].

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The increase in CO_2 concentration inside leaves promotes the closure of stomata, which may occur in response to abiotic stress [38]. This CO_2 concentration may be directly related to the increase in transpiration, which was greater than 0,006 mmol H_2O $m^{-2}s^{-1}$ (Fig. 3B). According to [39], the increase in resistance to gas diffusion can be a limiting factor in the CO_2 assimilation rate. The increase in transpiration by plants is mainly due to the inability of some plants to absorb enough water to replenish that consumed in the transpiration process [40], and the loss of water by plants is regulated by the activity of the guard cells [41]. As temperature rises, relative air humidity decreases and responses of metabolic processes in plants will reflect the interaction between transpiration and guard cell activities [42].

The efficiency in the use of water by sweet pepper plants demonstrates a relationship between photosynthesis and transpiration in which the observed values are directly related to the amount of carbon that the plant fixes for each unit of water it loses [43]. In this sense, decreases observed in water use efficiency (Fig. 3E) are reflective of increases in the rate of carbon dioxide assimilation and transpiration of plants. As for the instantaneous efficiency of carboxylation (Fig. 3F).

The results obtained in this work indicate that the increase in the instantaneous efficiency of carboxylation is related to the increase in the concentration of CO_2 and to the gains related to the rate of assimilation of CO_2. [44] ~~point~~Point out that this efficiency is related to the intercellular CO_2 concentration and the rate of assimilation of CO_2. The CO_2 assimilation from the external environment promotes water loss, which restricts CO_2 entry [41]. The gas exchanges, according to [45], are influenced by climatic conditions, so the reduction in the efficiency of water use may be related to the increase of solar radiation, temperature and relative humidity. [46], found a mean value of 0.28 mol of H_2O $m^{-2}s^{-1}$ for stomatal conductance in sweet pepper plants cultivated in protected environment, which is in agreement with the observed in this work (Fig. 3C).

It is noteworthy that the stomatal behavior determines the transpiratory requirement of the plants, thus controlling the loss of water in the form of vapor [47]. Although Si is not considered an essential element for plants, studies show that its application to the soil contributes to the growth and increase of productivity [48], as can be observed in this work (Table 3). In saline stress conditions, the plant growth is compromised due to the reduction of the osmotic potential of the soil solution, which reduces the water potential of the plants [49]. According to [50], this reduction of the water potential of the plants can be mitigated by the application of Si, which reduces the toxicity caused by excess sodium chloride in the soil solution.

The use of calcium silicates in the soil provides significant responses of the crops in the increase of the contents and P and in the reduction of the heavy metal content, resulting in a greater productive stability [51].

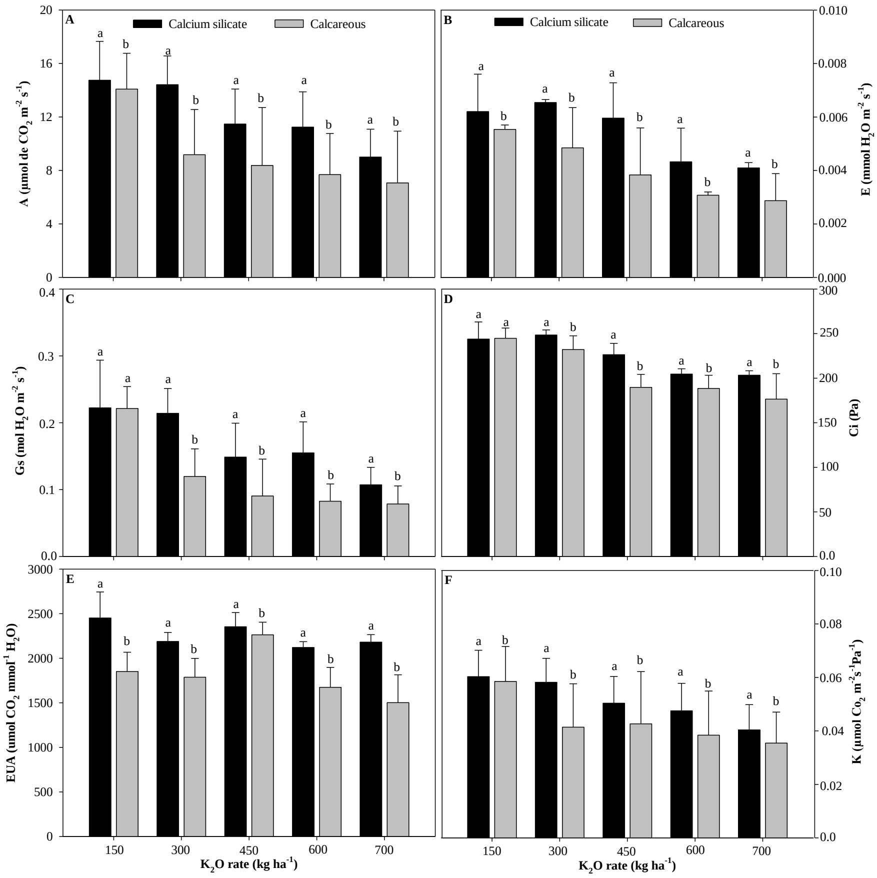

Fig. 3. Liquid photosynthesis (A), transpiration (B), stomatal conductance (C), intercellular CO_2 concentration (D), water use efficiency (E) and instantaneous efficiency of carboxylation (F) as a function of presence and absence of calcium silicate and doses of K_2O.

The production and weight of sweet pepper fruits were higher when the 150 kg K_2O dose was applied in the presence and absence of calcium silicate (Fig. 4A and 4B). Higher doses of K_2O reduced chilli fruit production (Fig. 4A) and caused a significant decrease in plant height (Fig. 4E). There was a reduction in the length of the chili fruits when the

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K_2O doses increased, in the presence and absence of calcium silicate (Fig. 4C). The application of calcium silicate favored the increase of the diameter of the fruits in the doses of K_2O studied (Fig. 4D). The beneficial effects of Si on the growth have been reported in a wide of plant species, which are characterized by protecting the plant from various biotic and abiotic stresses [52]. Transporters responsible for Si unloading from xylem in leaves also have been identified in many plant species [53]. The aerial plant parts accumulate more Si than roots [54]. Deposition of Si takes place in different parts of plant such as epidermis of shoots but can also occur in the cell wall of root endodermis [14]. However, phytoliths formation, composition, and localization vary among plant species [55].

The dose of 150 Kg K_2O favored the growth of sweet pepper plants in the presence of calcium silicate. In Fig. 4C it is observed that, as increasing doses of K_2O were applied, there was reduction in fruit length, as observed by [56]. Under conditions of higher salinity and osmotic pressure of the soil solution the absorption of water from the root cells decreases, allowing the occurrence of ionic toxicity. The addition of 16.6 g KCl m^{-2} reduced root yield and P uptake by sweet pepper plants cultivated on an Oxisol with 24.0 g dm^{-3} of organic matter [6] in addition, [57] reported that high salinity promotes changes in photosynthesis (CO_2 assimilation, stomatal conductance and leaf transpiration), thus inhibiting plant growth and reducing its height, as shown in Fig. 4E.

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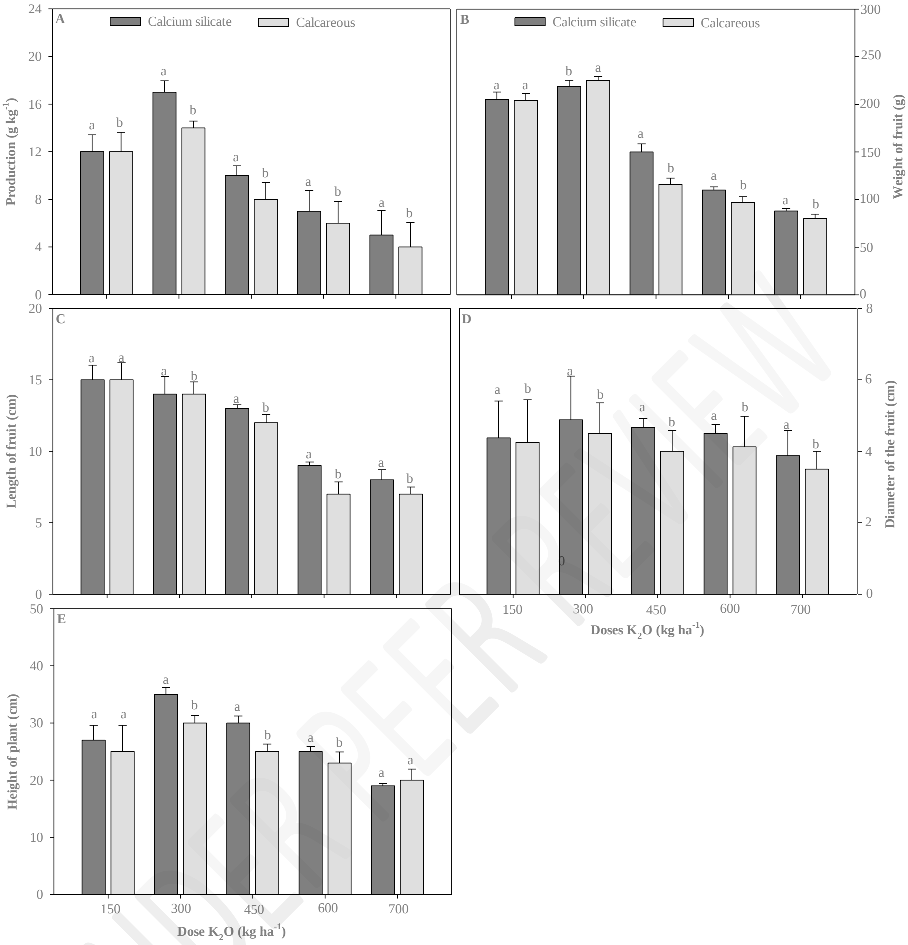

Fig. 4. Production (A), fruit weight (B), fruit length (C), fruit diameter (D) and plant height (E) as a function of the presence and absence of calcium silicate and K_2O doses.

4. CONCLUSION

It is concluded that, in the culture of sweet pepper in protected cultivation, the increase in the rates of potassium fertilization increases the electrical conductivity of the soil and the silicon content in the leaves. Doses up to 300 kg K_2O ha^{-1} increase the biometric factors of production. High doses of K_2O reduce gas exchange and water use efficiency, and the application of calcium silicate in the soil attenuates these effects.

COMPETING INTERESTS

We declared that no competing interests exist.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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